

Dive, food, and exercise effects on blood microparticles in Steller sea lions (*Eumetopias jubatus*): exploring a biomarker for decompression sickness.

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Abstract

Recent studies of stranded marine mammals indicate that exposure to underwater military sonar may induce pathophysiological responses consistent with decompression sickness (DCS). However, DCS has been difficult to diagnose in marine mammals. We investigated whether blood microparticles (MPs, measured as number/ μ l plasma), which increase in response to decompression stress in terrestrial mammals, are a suitable biomarker for DCS in marine mammals. We obtained blood samples from trained Steller sea lions (*Eumetopias jubatus*, 4 adult females) wearing time-depth recorders that dove to predetermined depths (either 5 or 50 m). We hypothesized that MPs would be positively related to decompression stress (depth and duration underwater). We also tested the effect of feeding and exercise in isolation on MPs using the same blood sampling protocol. We found that feeding and exercise had no effect on blood MP levels, but that diving caused MPs to increase. However, blood MP levels did not correlate with diving depth, relative time underwater, and presumably decompression stress—possibly indicating acclimation following repeated exposure to depth.

Key Words: Sea Lion; Decompression, Stress, Apnea, Diving, Bubbles

Introduction

The purpose of this study was to improve understanding of physiological responses to diving in a breath-hold diving marine mammal—with a particular focus on the pathophysiology of decompression sickness (DCS). DCS is a systemic pathophysiological process that occurs naturally after tissues become supersaturated with nitrogen under high atmospheric pressure. In humans, DCS can also occur when nitrogen or some alternative gas is used to dilute O₂ in breathing mixtures during activities such as deep-sea diving, high altitude aviation and space exploration. Most studies of DCS are performed with terrestrial mammals. We therefore hypothesized that a comparative physiological approach studying *bona fide* diving mammals would add valuable insight into the physiology of decompression. Such an approach may yield insights into the potential physiological traits that allow breath-hold diving animals to perform prolonged apneas to great depths without apparent pressure-related problems.

There are data indicating that diving mammals may sustain DCS, at least when they are subjected to atypical stresses (11, 16, 17). Recent necropsy reports have suggested a link between mass stranding of beaked whales and the use of naval mid-frequency sonar (10). The whales experienced symptoms that were similar to those caused by inert gas bubbles in human divers (4). These reports have increased the concern that anthropogenic sound, such as that created by military sonar or during seismic exploration, may harm marine animals. Specifically, it has been suggested that alteration in physiology or diving behavior may increase the risk of DCS (3, 15). For instance, blood bubble formation has been noted in some turtles that were trapped underwater and hauled rapidly to the surface. Those that received recompression treatment recovered and were released, thereby confirming a clinical diagnosis of DCS in a

diving vertebrate (12). Bubble formation is believed to be a crucial event in the etiology of DCS, but the role bubbles play in the disease process remains unclear (22). As more is learned about DCS, it has become apparent that some of the symptoms are similar to those of other disease states (18, 35, 36).

There are a few well-defined risk factors that increase the probability of DCS, such as increasing dive duration, dive depth, ascent/decompression rate, body mass, and breathing gas (8, 19, 20, 37). Recent studies have shown that microparticles (MPs) are elevated with decompression stress (31, 32). Microparticles are cellular fragments between 0.3 and 1 μm in size that are shed from various cells. MPs derived from platelets are known to activate leukocytes and cause aggregation, and can stimulate pro-inflammatory cytokines. MPs derived from decompression stress have been shown to specifically activate neutrophils and cause vascular damage (32). Microparticles correlate with depth of diving in mice (32), but correlate poorly with depth in humans (31, 33). Studies have also evaluated the effect of exercise on bubble and MP production in human scuba divers. Exercise can increase the number of circulating MPs slightly, whereas diving has a much greater effect and is also impacted by the gas used during the dive (31).

The question of potential causal relationships between bubbles, MPs, platelet-neutrophil interactions, and neutrophil activation remains obscure (30). Our study aimed to examine the relationship between decompression stress (depth and time underwater) and MP levels in Steller sea lions in a controlled diving experiment, in the context of diving, feeding, and exercise. We specifically chose this species as they generally dive to between 30-40 m (24), they inhale before diving (Fahlman et al, unpublished observation), and have been trained to safely perform

repeated breath-holds without experiencing symptoms of gas bubble disease during dives up to 50 m (6). In addition, a theoretical gas dynamic model (5) predicted that for 12 repeated dives to 50 m there would be a significant increase in blood and tissue N₂ levels. Thus, we hypothesized that repeated breath-hold foraging dives would increase blood MP levels, and that the levels would increase with diving depth and time underwater. To control for the potentially confounding effect of feeding and exercise while diving, we also measured blood MP levels following exercise and feeding in separate experiments.

It is important to recognize that while we introduce the study in the context of the potential for decompression sickness, the diving experiments described here represent decompression stress as opposed to overt decompression sickness. Increase and decrease in pressure is a biomechanical stressor, in that whenever a diving mammal ascends, the gas in the lung expands, gas solubility decreases, and the animal has to manage changes consequent to that stressor. Thus our use of ‘decompression stress’ is considered and accurate. Diving mammals usually dive without clinical impact, but they nonetheless manage decompression stress during every dive. The question of interest to us was how do they do it? Addressing this question furthers knowledge about the physiological and behavioral mechanisms of diving that interests basic science and may have conservation impacts.

Methods

Animals

All experiments were conducted under permits from the Animal Care Committees of the University of British Columbia and the Vancouver Aquarium. Four female Steller sea lions

(*Eumetopias jubatus*) trained to dive to fixed depths participated in 3 dive experiments (Table 1). Animals were weighed the day before a dive experiment, and had not been diving for at least 4 days prior to the first dive experiment. Each experiment was then separated by at least 2 days.

Dive experiments

The general experimental arrangement was as described previously (6, 9), with sea lions trained to dive on command from within a respiratory dome to a specific depth. The depth was defined by the length of a feeding tube delivering food to the end of the tube to reinforce the behavior. Dive experiments were repeated 3 times each at depths of 5 m and 50 m. All dive experiments were conducted in June.

For each experiment, pre-treatment blood samples were taken the morning of the dive before the animal had been fed. Samples were drawn directly into 5 ml Cyto-Chex BCT vacutainer tubes (Streck, Omaha, NE). To minimize shearing of blood components, care was exercised to obtain a clean venepuncture and good sample flow. The sea lion was then transported to the dive site and allowed to dive for a pre-determined duration of approximately 30 min.

Blood samples were again taken 3 and 24 hours after the sea lion had surfaced after the last dive. Collecting blood samples immediately following the dive was deemed unsafe for both animals and personnel. A 3 hr delay was therefore deemed acceptable before collecting the first blood sample. The 24 hr post-dive sample was based on data in terrestrial mammals and allowed us to trace the time-course for changes in blood MP levels (32). Food was offered during the dive and

after each blood sampling to reinforce behaviors. The same sampling protocol was repeated with only surface swimming and with feeding without access to the water.

Dive Dose

As the probability of DCS increases with both depth and dive duration, we computed a simple index, called dive dose, to estimate the relative time underwater for each experiment. Dive dose was estimated by integrating the dive depth (m) over time (sec) to calculate the index for each dive, which represents the total “depth exposure” for each animal while underwater.

Metabolic rate (rate of O₂ consumption)

The rate of O₂ consumption ($\dot{V}O_2$, L O₂ min⁻¹) before and during a dive was used as an index of the metabolic rate. The metabolic rate before and after the dive bout was assessed by measuring the gas concentrations in the metabolic dome (6). We separated metabolic rate into surface metabolic rate before diving (pre-dive), at which time the sea lion had received minimal amount of food and was post-prandial. As both the dive and surface interval durations were determined by the sea lion, and as reliable estimates for the metabolic rate of individual dives within a dive bout cannot be made (6, 7, 9), we computed the diving metabolic rate for the entire diving bout. Specifically, we divided the total volume of O₂ taken up from the beginning of the first dive until the end of the post-dive recovery period by the duration of that same period. Thus, we only computed one pre-dive and one diving metabolic rate for each experiment for each sea lion.

Exercise and feeding experiments

Two separate experiments were conducted to evaluate the potential confounding effect of exercise or feeding/digestion on blood MP levels. For the exercise experiments, the sea lions performed a 30 min surface swim by following a boat driving at a speed (approximately $2 \text{ m} \cdot \text{sec}^{-1}$) similar to the estimated ascent and descent rates and underwater swim speed during the dive experiments. Each sea lion performed two exercise experiments. For the feeding experiment, the animals received a meal of the same size as during the dive experiments over a 30 min period. For both experiments, a blood sample was taken before and after (3 hr and 24 hr) the experiments. Thus, we tried to replicate the time course used during the experiment to separately assess the effect of feeding, digestion, and exercise on blood MP levels.

Blood MP levels

All blood samples were taken by venepuncture using a butterfly needle and a vacutainer. Blood MP levels were measured using flow cytometry as previously described (31, 32). Microparticle concentration data were transformed to a relative change from the pre-dive value (control) such that positive values indicate an increase ($(\text{MP}_{\text{post-dive}} - \text{MP}_{\text{control}}) / \text{MP}_{\text{control}} \times 100$). We analyzed the relationship between a dependent variable and three different experimental variables (time after dive, depth, and the product of time and depth, i.e., dive dose) using linear-mixed effects models (lme, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, version 3.1.0, 2014). Individual animal was treated as a random effect, which accounted for the correlation between repeated measurements on the same individual (21). We used the Akaike information criterion (AIC) to select nested models. In this study P-values ≤ 0.05 were considered as significant and $P \leq 0.1$ were considered a trend. Data are presented as the mean \pm standard deviation (SD), unless otherwise stated.

Results

Animals

Four female Steller sea lions participated in 3 dive experiments and the average weight and ages are summarized in Table 1. Animals were weighed the day before a dive experiment and each experiment was separated by at least 2 days.

Dive behavior, metabolic rates and dive dose

The dive metabolic rates were significantly higher for both dives to 5 m ($3.22 \pm 0.73 \text{ L O}_2 \text{ min}^{-1}$, paired t-test, $P < 0.05$, $t = 4.42$, $df=3$) and 50 m ($3.03 \pm 0.63 \text{ L O}_2 \text{ min}^{-1}$, $P < 0.05$, $t = 2.81$) as compared with the pre-dive metabolic rates (5 m: $2.15 \pm 0.50 \text{ L O}_2 \text{ min}^{-1}$; 50 m: $2.35 \pm 0.41 \text{ L O}_2 \text{ min}^{-1}$). There were no differences in metabolic rate for dives to 5 m as compared with 50 m ($t_{10} = 0.47$, $P > 0.6$, Table 1). The average dive duration and inter-dive surface interval for dives to 5 m (dive duration: $50 \pm 11 \text{ sec}$; surface interval: $11 \pm 3 \text{ sec}$) were significantly lower than dives to 50 m (dive duration: $105 \pm 21 \text{ sec}$, $t_{10} = 4.49$, $P < 0.01$; surface interval: $35 \pm 7 \text{ sec}$, $t_{10} = 6.86$, $P < 0.01$, Table 1).

The average dive dose ($\text{m} \cdot \text{sec}$) was significantly lower for the 5 m (T1, $7868 \pm 264 \text{ m} \cdot \text{sec}$) as compared with either the first (T2, $50848 \pm 7206 \text{ m} \cdot \text{sec}$) or second (T3, $44729 \pm 6107 \text{ m} \cdot \text{sec}$) dive experiment to 50 m ($P < 0.01$, paired t-test). In addition, dive dose was significantly higher during the first dive experiment to 50 m as compared with the second ($P < 0.05$, paired t-test, T2 vs T3)

Blood MP levels

There was a significant difference in pre-dive blood MP levels between experimental trials ($AIC_{\text{null}} = 192.6$, $AIC_{\text{time}} = 151.5$, $P < 0.01$). There was a trend toward a 26% decrease in MP levels from the first (2604 ± 352 MPs ul^{-1} , 5 m trial, Fig. 1) to the second experiment (1935 ± 163 MPs ul^{-1} , $P < 0.1$, 50m Trial 1) and then a significant 57% increase from the first to the last experiment (4082 ± 788 MPs ul^{-1} , $P < 0.05$, 50m Trial 2).

There was a significant increase in MP levels following a dive ($AIC_{\text{null}} = 485.7$, $AIC_{\text{time}} = 446.0$, $P < 0.01$). Blood MP levels had increased by 170% 3 hours following a dive bout and by 536% after 24 hours (Fig. 1). There was a trend for an increase with dive depth ($AIC_{\text{time}} = 478$, $P < 0.1$), but this was mainly because of the much higher MP levels at 3 hours following the first 50 m dive experiment (Fig.1). Feeding without diving increased the blood MP levels by 24% 3 hours after feeding ($AIC_{\text{null}} = 126.0$, $AIC_{\text{fed}} = 110.4$, $P < 0.01$), and the levels were back to control levels after 24 hours.

The effect of exercise on MP levels were tested twice and both times the pre-exercise MP levels (control) were much more variable and higher than any blood samples collected either before or after diving (range MP levels after diving: 1763 - 24830 MPs ul^{-1} , Max levels before exercise: 18405 - 778750 MPs ul^{-1}). The reason for this is unclear and warrants further investigation. While there was a significant change in relative MP levels ($AIC_{\text{null}} = 357.3$, $AIC_{\text{time}} = 339.8$), this change occurred between the 3-hr and 24-hr post-exercise blood samples (81% increase, $P < 0.05$) and neither the MP levels at 3 hr or 24 hr were different from the pre-exercise levels ($P >$

0.1, Fig. 1). Similarly, the MP levels increased following feeding (Pre-feeding: 2570 ± 108 MPs ul^{-1} , Post-feeding: 3175 ± 414 MPs ul^{-1} ; $P < 0.05$, $t_6=2.83$, Fig. 1).

Discussion

This experimental study of Steller sea lions indicates that, as is true of terrestrial mammals, MPs increase in response to diving. There is variability in baseline MPs numbers at the outset of diving studies, similar to observations with mice and humans, which indicates that stressors other than diving impact MPs levels. There was not a consistent increase in MPs with depth of a dive or dive dose (relative time and depth underwater). This finding is consistent with studies of human divers, but differs from results with inbred mice. More broadly, this raises the question whether measurement of MPs in sea lions can reflect decompression stress. We do not have a clear understanding of the reason for this; thus further experimental study is warranted to further define what variables affect blood MP levels.

It has been over 100 years since some of the first studies on human issues related to decompression following exposure to pressure were published (2). Since then, several studies have attempted to understand the etiology of this disease and to find ways to prevent, or at least significantly reduce, the risk of decompression sickness in human divers (e.g. 25, 37, 38). They have shown that the primary reason for development of clinical signs is due to an increasing inert gas burden (8, 37), with gas being released from solution as pressure and solubility is reduced during the decompression phase. Bubbles form in tissues or within the vasculature where they can block flow or initiate an inflammatory response (18, 35, 36).

245

246 Several physiological changes occur following a decompression event. For example, a reduction
247 in platelet count or complement activation appear to correlate with the levels of decompression
248 stress (26, 35). Circulating MPs also correlate with the magnitude of the decompression in
249 uniform 2-hour dives in mice (32), although more recent work with human scuba divers found no
250 correlation between MP elevations and depth of diving (29). It is notable in the human studies
251 that duration of a dive and relationship between eating and diving were not addressed.
252 Circulating blood MP levels may still be a useful tool to diagnose DCS, however, because
253 human divers with DCS have significantly higher levels of MPs with specific surface proteins
254 than asymptomatic, control divers (29).

255

256 We hypothesized that blood MP levels may be a good indicator of the level of decompression
257 stress in breath-hold diving mammals given that MP levels indicate decompression stress in
258 divers breathing pressurized air. We therefore allowed Steller sea lions to dive repeatedly to two
259 different depths, 5 m and 50 m, assuming that dives to 50 m would cause a greater
260 decompression stress. However, we found high variability in the circulating blood MP levels of
261 the animals diving to 50 m (T2 vs T3, Fig. 1). The risk of DCS is not only affected by dive depth
262 alone, but dive duration and ascent rate also alters the risk (37), and it has been shown that only a
263 5% change in the inert gas burden can result in a 50% change in the probability of DCS (8).

264

265 We used the integrated depth as a simple estimate of decompression stress (dive dose). The dive
266 dose was significantly greater for deeper dives, but the dive dose was also significantly greater

for the first dive (T2) to 50 m as compared to the second (T3). This may explain the lower circulating MP levels during T3 as compared with T2, but not the shallower dive. The responses during breath-hold diving versus scuba diving may be different. For example, the breath-hold dives included animals foraging, exercising, and with increasing levels of hypoxia. This may cause a greater variability in the response as compared with experimental dives on scuba diving humans or animals in a pressure chamber that are continuously breathing.

Mild oxidative stress and hypoxia increase MP levels, and it is also possible that higher activity levels during shallow dives elevate blood MP levels (1, 34). During breath-hold diving, there is a rapid and short period of hyperoxia when the lungs compress and the pulmonary gas pressure increases and gas diffusion continues. Blood and tissue PO_2 continuously decrease throughout most of the dive, with levels < 10 mmHg commonly reported in diving California sea lions (23). Thus, while dive duration and end-dive PO_2 correlate, there was considerable variability between dives and individuals (23). As sea lions are generally quite active while diving, this variability could reflect variation in underwater activity.

The dive pattern for the Steller sea lions in our study was similar to those previously reported for the same animals (7, 9, 13, 14, 27, 28), where deeper dives were longer and associated with longer surface durations to replenish the O_2 stores. While there were no significant differences in the metabolic rate over an entire dive bout (Table 1), the diving metabolic rate correlated with underwater activity, and the activity was generally greater for shallower dives (7, 9). Thus, shallow dives are shorter, but of higher intensity—while the deep long dives are prolonged

events of lower intensity. Consequently, the sea lions may have become more hypoxic during the high intensity shallower dives, which could have independently increased MP levels.

There is anecdotal evidence suggesting that repeated hyperbaric exposures help to reduce DCS incidence, and a controlled study showed that rats acclimated over as little as 4 days showed reduced DCS incidence compared with control animals (25). In our study, the sea lions had not been diving actively for at least 4 days before the first dive experiment. The active diving to 5 m (T1, Table 1) appeared to increase blood MP levels for at least 24 hrs, which then returned to pre-dive levels prior to the 2nd experiment (Fig. 1). The blood MP levels again increased during the first dive experiment to 50 m, at levels that exceeded the 5 m dives (Fig. 1). However, the pre-dive MP levels did not return to control levels between the first and second dive to 50 m (T2 vs T3, table 1), suggesting that the 48 hr recovery time between experiments may not have been sufficient. Thus, while the pre-dive MP levels showed no systematic pattern before diving the animals may have become acclimated and less responsive to decompression stress, similar to that observed in rats (25).

There are other possible reasons that the relationship between blood MP levels and decompression stress (time and depth) did not correlate. Using blood MP as a biomarker is a relatively new concept and as this is the first study to look at how blood MP levels change in a diving mammals, we undertook separate experiments to determine the effect of digestion or exercise on blood MP levels. For the former, we gave the sea lions a meal of similar size to what they caught during diving. For exercise, we had the sea lions perform a surface swim running at

a similar speed that we estimated the sea lions were swimming underwater while foraging. Neither of these variables seemed to significantly change the MP levels above the control levels (Fig. 1).

In summary, blood MP levels seem to be a useful biomarker to identify decompression stress. However, the magnitude of decompression stress (dive dose or depth) correlated poorly with blood MP levels in Steller sea lions. We suggest that hypoxia or acclimation may affect the response to decompression stress during breath-hold diving, which may explain the variation in biomarker levels for dives to different depths. We conclude that neither digestion nor exercise affected the blood MP levels and should not have influenced our findings. Thus, blood MP levels may be a useful index to identify decompression stress, but more research is needed on Steller sea lions and other species of marine mammals to verify our findings and separate the potential confounding effects of hypoxia and acclimation.

Perspectives and Significance

Marine mammals are diverse group of animals with over 100 species that obtain their food from the ocean. Million of years of evolution have enabled them with physiological traits to master the art of free diving, and avoid the problems that increasing pressure have on physiological homeostasis. These animals appear to avoid the risk of DCS during natural dives, but recent studies have shown that under certain circumstances departure from normal physiology or behavior may cause bubbles to form. While the diving physiology of marine mammals has been studied for well over 70 years, this area of their physiology has received relatively limited attention. To improve understanding about the potential traits that reduce DCS risk, we investigated changes in blood MP levels during a natural diving bout in Steller sea lions. The

results help improve basic understanding about their physiology, and are important to assess the potential ecological impact of these species to changes in the environment. Thus, comparative studies on the diving physiology of marine mammals may have significant relevance for conservation efforts.

Authors' contributions

AF and MM conceived of the study, designed the experiments, obtained funding, collected and analyzed the data, carried out the statistical analysis, and drafted the paper; AWT, DR participated in the design of the experiments and data collection, provided access to trained animals, helped draft the manuscript; MH participated in the design of the experiments, provided veterinary support during anesthesia, participated in data collection; NW and TN helped design experiments, participated in data collection and were in charge of animal training; MY helped with microparticle analysis; SRT helped design experiments and performed analysis of microparticle levels. All authors gave final approval for publication.

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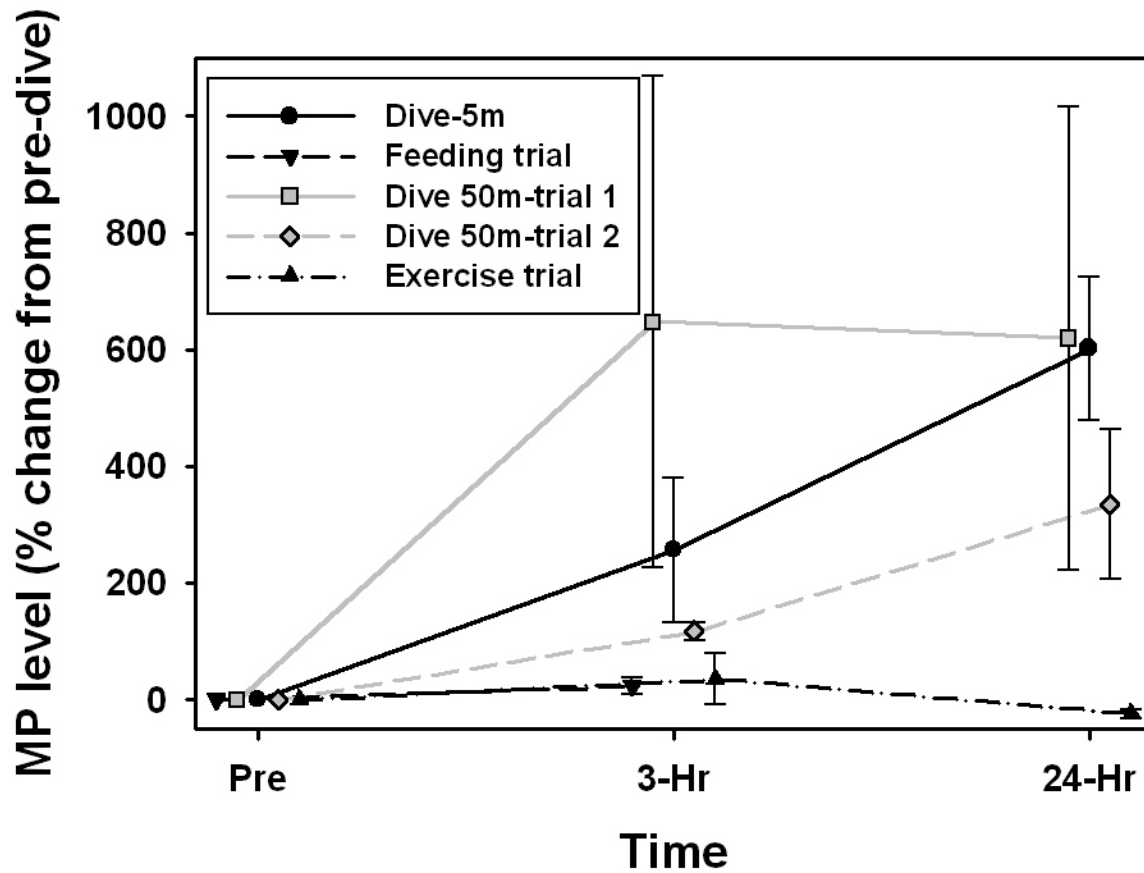
Table 1. Animal ID, age, body mass, and average dive duration (number in parenthesis is number of dives), and measured pre-dive and dive metabolic rate for each trial (T1-5 m dive, T2-1st 50m dive, T3-2nd 50 m dive). The pre-dive metabolic rate was the measured oxygen consumption rate ($\dot{V}O_2$, L O₂ min⁻¹) during the last 2-3 min in the respirometry dome before diving. Dive metabolic rate was the cumulative volume of O₂ consumed from after the first dive until the end of the post-dive recovery period divided by the time from the start of the first dive until the end of the post-dive recovery period (6).

Animal	Age (years)	M_b (kg)	Dive duration (sec)			Surface interval (sec)			Pre-dive $\dot{V}O_2$ (L O ₂ min ⁻¹)			Dive $\dot{V}O_2$ (L O ₂ min ⁻¹)		
			T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
F97H A	15	171±	47±	135±	120±	11±	44±	42±	2.67	2.59	2.88	3.38	2.16	2.91
		1	17 (31)	48	47	4	14	11						
F00B O	12	160±	47±	122±	105±	14±	38±	30±	1.54	1.76	1.70	2.59	2.33	2.62
		1	15 (29)	19	8	12	9	6						
F97S I	15	229±	39±	100±	102±	7±3	31±	34±	2.42	2.59	2.48	4.18	3.22	3.37
		2	12	21	13		12	5						

			(38)											
F00Y	12	204±	66±	85±1	71±2	11±	24±	35±	1.95	2.32	2.44	2.71	3.71	3.88
A		1	9	8	2	3	12	23						
			(23)											
Gran	13.5	191±	50±	111±	100±	11±	34±	35±	2.15±0	2.32±0	2.38±0	3.22±0	2.86±0	3.20±0
d	±1.7	32	11	22	21	3	9	5	.50	.39	.49	.73	.74	.55
mean														

372

373 Figure 1. Mean (\pm SD) blood microparticle levels before, and 3 hours or 24 hours following a
374 dive or exercise bout, or before and 3 hours following a feeding event. Dive bouts were either to
375 5 m or 50 m and the latter was repeated twice (see text). All data points are samples from 4 sea
376 lions except 2nd dive trial to 50 m at 3 and 24 hours.



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